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(54) Title: METHOD OF IDENTIFYING MHC-CLASS I RESTRICTED ANTIGENS ENDOGENOUSLY PROCESSED BY A CELLULAR SECRETORY PATHWAY

(57) Abstract

A method of identifying antigens which are capable of being endogenously processed by a cellular secretory pathway comprising: introducing an antigen into a donor cell lacking in MHC class I molecules, incubating in an *in vitro* medium the donor cells, primed cytotoxic T lymphocytes having specificity for the antigen, and target cells which express MHC class I molecules and are labelled with a detectable intracellular marker, under suitable conditions such that the donor cells remain intact, and measuring the amount of detectable marker released into the incubation medium. A method of assaying a medium for the presence of a substance that affects processing of an endogenously processed antigen in a cellular secretory pathway; and a method of characterizing a tumor or viral antigen capable of being endogenously processed by a cellular secretory pathway.

Title: METHOD OF IDENTIFYING MHC-CLASS I RESTRICTED ANTIGENS ENDOGENOUSLY PROCESSED BY A CELLULAR SECRETORY PATHWAY

FIELD OF THE INVENTION

5   The invention relates generally to a method of identifying antigens which are capable of being endogenously processed by a cellular secretory pathway and to a method of assaying a medium for the presence of a substance that affects processing of an endogenously processed antigen in the cellular secretory pathway. Further, the invention relates to a method of characterizing cytotoxic T cell antigens capable of being endogenously

10   processed by the cellular secretory pathway.

BACKGROUND OF THE INVENTION

The cytotoxic T lymphocyte (CTL) response is a major component of the immune system, active in immune surveillance and destruction of infected or malignant cells and invading organisms expressing foreign antigens on their surface. The ligand of the

15   antigen-specific T lymphocyte receptor is a complex made up of a peptide fragment of a foreign antigen 8 to 10 amino acids in length, presented in the groove of MHC class I molecules. Unlike B cells, T cells do not recognize intact native antigen molecules. In general, cytotoxic T cell activation requires that the antigen be processed endogenously and cleaved into specific peptide fragments which are presented on the surface of antigen

20   processing cells in association with class I MHC molecules.

MHC class I molecules are normally expressed at the cell surface as ternary complexes formed by a heavy chain of 46 kD, a light chain called β_2-microglobulin (β_2m) of 12 kD and a peptide composed of 8-10 amino-acids (van Bleek, G. M. and S. G. Nathenson, *Nature* 348:213, 1990; Zhang, W. et al., *Proc. Natl. Acad. Sci. USA* 89:8403, 1992;

25   Matsumura, M. et al., *Science* 257:927, 1992; and Latron, F., et al., *Science* 257:964, 1992). Formation of the ternary complex is thought to involve transport into the lumen of the endoplasmic reticulum (ER) of peptides generated by protein degradation in the cytoplasm (Nuchtern, J.G. et al., *Nature* 339:223, 1989; Yewdell, J. W. and J. R. Bennink, *Science* 244:1072, 1989; and Cox, J.H. et al., *Science* 247:715, 1990). The study of mutant cell lines

30   selected for their low expression of MHC class I molecules at the cell surface has provided insights into the molecular events required for antigen processing. These studies have allowed the identification of two genes located in the MHC region which encode proteins of the ATP binding cassette (ABC) family. These genes, called TAP-1 and TAP-2, have been implicated in transport of peptides from the cytoplasm to the lumen of the ER (Deverson,

35   E.V. et al., *Nature* 348:738, 1990; Trowsdale, J. et al., *Nature* 348:741, 1990; Spies, T. et al., *Nature* 348:744, 1990; Monaco, J.J. et al., *Science* 250:1723, 1990; Spies, T. and R. DeMars, *Nature* 351:323, 1991; Bahram, S. et al., *Proc. Natl. Acad. Sci. USA* 88:10094,1991; Spies, T. et al., *Nature* 355:644, 1992; Kelly, A. et al., *Nature* 355:641. 1992; Powis, S. H. et al., *Proc.*

Natl. Acad. Sci. USA 89:1463, 1992; and Colonna, M. et al., *Proc. Natl. Acad. Sci. USA* 89:3932, 1992). Two other MHC linked genes, LMP-2 and -7 (Monaco, J.J. and McDevitt, 1982, Proc. Natl. Acad. Sci. USA 79:3001), are components of the proteasome, a cytoplasmic multicatalytic protease complex, which is likely responsible for some aspects of protein

5   degradation for antigen processing (Ortiz-Navarette, V. et al., *Nature* 353:662, 1991; Brown, M. G. et al., *Nature* 353:355, 1991; Glynne, R. et al., *Nature* 353:357, 1991; Martinez, C.K. and J.J. Monaco, *Nature* 353:664, 1991; Kelly, A. et al., *Nature* 353:667, 1991; Yang, Y. et al., *Proc. Natl. Acad. Sci. USA* 89:4928,1992;Goldberg, A.L. and K.L. Rock, *Nature* 357:375, 1992).

CTL recognize MHC class I molecules bearing endogenously processed foreign

10   or aberrant self proteins, thereby enabling the immune system to respond to intracellular pathogens or tumor transformation. The endogenous cellular processing pathways of MHC class I antigen presentation is thought to require that the antigen be expressed endogenously in the cytoplasm or ER of the presenting cell. The endogenous peptides associate with nascent membrane bound MHC class I molecules before they leave the ER and are eventually

15   presented at the cell surface bound to the MHC class I molecules (Yewdell, W. and J. R. Bennink, 1989, Science 244:1072).

Peptides unable to bind MHC Class I because they are in excess or lack the correct MHC binding motif for the MHC haplotype are thus far undetectable by the techniques commonly used in the field and are presumed to be short lived and degraded

20   (Falk et al., *Nature*, 348:248-251, 1990; Rötzschke et al., *Nature*, 348:252-254, 1990). But recent results (Arnold et al., *J. Exp. Med.*, 182:885-889, 1995) suggest that peptides not able to bind to a MHC class I molecule intracellularly may be found bound to heat shock proteins (HSPs) such as gp96. These authors show that cellular antigens are represented by peptides associated with gp96 molecules independently of the MHC class I expressed confirming

25   earlier results by Udono and Srivastava. (*J. Exp. Med.*, 178:1391-1396, 1993; *J. Immunol.*, 152:5398-5403, 1994). Gp96 extracted from a specific cell are able to induce "cross-priming".

Huang et al. (*Science*, 264:96-965, 1994) have elucidated mechanisms in which proteins or peptides may either be exchanged from donor to recipient after release from the cell surface or released following uptake of antigen by the antigen processing donor.

30   Though these phenomena may operate under specific circumstances, they relate to the processing of exogenous antigens rather than endogenous antigens.

Autoimmune disease results from the misdirection of the immune response to self antigens resulting in host tissue damage. Autoimmune diseases may be triggered by infectious agents, such as bacteria and viruses, environmental conditions and certain drugs

35   and toxins. Several mechanisms have been put forward which may account for some, but not all of the features of autoimmunity. For example, autoimmunity may result from the break down of thymic self tolerance mechanisms the mimicry of self proteins by pathogen proteins and from the degeneration of peripheral T cell tolerance.

Although the exact mechanisms are poorly understood, the generation of autoimmune lesions is thought to require an MHC susceptibility allele capable of binding and presenting the antigens that initiate the autoimmune process; reactive T cells specific for those antigens and; target antigens available for presentation by an MHC susceptibility

5 allele to antigen specific T lymphocytes (Sinha, A.A. et al. 1990, *Science* 248:1380). Immune responses initially directed against microbial pathogens are implicated in some autoimmune diseases and autoantibodies specific for a self antigen often also have specificity for viral or bacterial determinants. The mechanisms behind this pathogen-associated autoimmune injury are poorly understood and the sites of autoimmune damage resulting from pathogenic

10 infection are often observed at sites distant from the site of infection. The identification of the target antigens of the autoimmune response may permit the rational development of therapies to block the interaction of these antigens with the autoimmune response, for example by the specific deletion of the anti-self T lymphocytes involved. Accordingly, there is a need for an assay which can be used to identify the target antigens which are

15 recognised by CTL in the generation of an autoimmune response.

CTL also play an important defensive role in the mammalian immune response to destroy invading pathogens, such as bacteria, viruses and microorganisms and defective cells, such as cancer cells. There is a need for an assay which can be used to identify those epitopes of foreign pathogens, precancerous cells and cancer cells which are

20 recognised by CTL. Synthetic peptides have been used in attempts to identify viral and tumor target epitopes recognised by CTL. However, misleading results have been obtained as, frequently CTL may recognize only very short segments of the synthetic peptide. The acid elution method has been used in attempts to identify the short epitopes presented on the cell surface in association with MHC. The epitopes, once identified, may form the

25 basis for the design of therapeutics for T lymphocyte mediated autoimmune disease, pathogenic infections and tumors. In particular, it is expected that the epitopes will be useful for as the basis for vaccines to specifically prime CTL.

SUMMARY OF THE INVENTION

The present inventors have surprisingly found that antigens may be

30 endogenously processed and secreted by the secretory pathway of a cell. The secreted antigens were shown to associate with cell surface MHC class I molecules of bystander target cells.

The present inventors found that virally infected cells or cells transfected with a viral antigen were able to transfer endogenously processed peptides *in vitro* to

35 recipient target cells for presentation to, and efficient recognition by, primed cytotoxic T lymphocytes (CTL) specific for the virus. Importantly, the present inventors demonstrated that the peptide transfer did not involve viral killing or CTL liberation of peptides from

infected cells or the release of membrane-bound MHC class I associated antigen from the cell surface.

Peptide transfer by the virally infected cells was found to be dependent on the presence of TAP transporters. Surprisingly, peptide transfer was found to be independent of the presence of MHC class I molecules, showing that the transferred peptide is not being transported to the cell surface in association with membrane bound MHC class I molecules. Peptide transfer was shown to occur through active transport via the secretory pathway of the cell and an inhibitor of the secretory pathway was shown to inhibit peptide transfer to target cells.

The invention therefore contemplates a method of identifying antigens which are capable of being endogenously processed by a cellular secretory pathway and transferred to bystander target cells for presentation to cytotoxic T lymphocytes, comprising: introducing an antigen into a donor cell lacking in MHC class I molecules; incubating in an *in vitro* medium the donor cells, primed cytotoxic T lymphocytes having specificity for the antigen, and target cells which express MHC class I molecules and are labelled with a detectable intracellular marker, under suitable conditions such that the donor cells remain intact, and; measuring the amount of detectable marker released into the incubation medium.

Preferably the antigen is a viral, bacterial or tumor antigen. In an embodiment the antigen is VSV, an allo peptide or a self peptide.

The invention also relates to a method of assaying a medium for the presence of a substance that affects processing of an endogenously processed antigen in a cellular secretory pathway, comprising: incubating in an *in vitro* medium a donor mammalian cell having an antigen capable of being endogenously processed by a secretory pathway of the donor cell, a target cell expressing MHC class I molecules and labelled with a detectable intracellular marker, and primed cytotoxic lymphocytes having specificity for the antigen, in the presence or absence of a substance that is suspected of affecting processing of endogenously processed antigen in the cellular secretory pathway, under suitable conditions such that the donor cells remain intact, and; measuring the amount of detectable marker released into the medium in the presence or absence of the substance.

In an embodiment, the donor cell lacks MHC class I molecules. Any cell expressing MHC Class I molecules may be used as the target cell. In a preferred embodiment, mammalian target cells, including human cells, may be used. In a particular embodiment the antigen is VSV and the target cell is RMA.

It is expected that substances that affect processing of an endogenously processed antigen in a cellular secretory pathway will be useful as immunomodulators for stimulating or inhibiting the immune response.

The invention still further relates to a method of characterizing a tumor or viral antigen comprising: obtaining, from a mammal, tumor cells or virally infected cells

and primed cytotoxic T lymphocytes having specificity for the tumor or virally infected cells; preparing a cDNA library from the tumor or virally infected cells and expressing the cDNA library in transfected host cells; incubating in a medium, transfected host cells, target cells expressing MHC class I molecules and labelled with a detectable intracellular marker

5 and the primed cytotoxic lymphocytes under suitable conditions such that the donor cells remain intact, and; measuring the amount of the detectable marker released into the medium and identifying those transfected cells capable of causing lysis of the target cells and characterizing the transfected DNA and expression product of the transfected DNA of the identified transfected cells. As will be appreciated, the method may also be employed to

10 characterize alloantigens, self antigens and antigens of other pathogens, such as bacteria, protozoa, or other microorganisms or parasites. Transiently transfected COS cells may be employed in the methods of the invention.

The present invention also contemplates the cloning of genes encoding the antigens characterized by the methods of the invention and vaccines incorporating the

15 characterized antigens and epitopes.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, reference is made herein to various publications, which are hereby incorporated by reference in their entirety.

20 **DESCRIPTION OF THE DRAWINGS**

The invention will be better understood with reference to the drawings in which:

Figure 1A is a bar graph showing the concentration of VSV-N peptide which yields a particular percentage of cytotoxicity;

25 Figure 1B is a bar graph showing the ability of VSV infected cells (RMA+VSV), cells pulsed with synthetic peptide (RMA+p) and control cells (RMA+-) to generate and transfer peptides to uninfected, chromium laden, target cells;

Figure 1C is a bar graph showing the ability of RMA cells infected for 12 hours (RMA + VSV), or treated with VSV immediately prior to adding the effectors

30 (RMA.4 + VSV) to generate and transfer peptides to uninfected, chromium laden, target cells;

Figure 2A is a bar graph showing the ability of human T1, or mouse Ltk-(L), and NIH3T3 (NIH) cells treated with or without (L+--) VSV to generate and transfer peptides to uninfected, chromium laden, target cells;

35 Figure 2B is a bar graph showing that T1, L, and NIH 3T3, cells were not lysed by VSV specific K^b restricted CTL;

Figure 2C is a bar graph showing ability of TAP 1 expressing CMT.1-4 cells to generate and transfer peptides to uninfected, chromium laden, target cells;

Figure 3A is a bar graph showing that VSV infected human T2 and mouse CMT.64 cells are not recognized by VSV specific K^b restricted CTL;

Figure 3B is a bar graph showing the effect of heavy and light MHC chains on peptide transfer using $\beta2$ m deficient R1E cells and R1E cells transfected with K^b(RIEKb), and R1E transfected with $\beta2$ m and K^b(R1EKbb2), with or without VSV treatment;

Figure 4 is a bar graph showing the effect of brefeldin A (BFA) on peptide transfer;

Figure 5 is a bar graph showing that supernatants from Ltk⁻ cells infected with VSV mediated lysis of RMA cells by VSV specific CTL;

Figure 6 is a diagram illustrating the surrogate antigen processing of the invention and alternative fate of antigenic peptides within the secretory pathway;

Figure 7A is a graph showing a CTL control assay from spleen cells not pulsed with VSV-N 52-59 peptide;

Figure 7B is a graph showing a CTL assay from spleen cells pulsed with VSV-N 52-59 peptide;

Figure 7C is a graph showing a CTL assay from spleen cells pulsed with VSV-N 52-59 peptide;

Figure 7D is a graph showing a CTL assay from spleen cells pulsed with VSV-N 52-59 peptide;

Figure 8A is a bar graph showing that peptide transfer occurs following infection with a recombinant vaccinia virus encoding the VSV-N 52-59 and is MHC haplotype independent; and

Figure 8B is a bar graph showing that peptide transfer occurs in a TAP dependent manner.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present inventors have demonstrated that antigens may be endogenously processed and secreted by the secretory pathway of a cell and cells are able to transfer endogenously processed peptides *in vitro* to recipient target cells for presentation to, and efficient recognition by, specifically primed CTL.

The present inventors demonstrated that the peptide transfer is not dependent on viral killing or CTL liberation of peptides from infected cells or the release of membrane-bound MHC class I associated antigen from the cell surface.

Peptide transfer by the virally infected cells was found to be dependent on the presence of TAP transporters. Surprisingly, peptide transfer was found to be independent of the presence of MHC class I molecules, showing that the transferred peptide is not being transported to the cell surface in association with membrane bound MHC class I molecules. Peptide transfer was shown to be occurring through active transport via the secretory

pathway of the cell and an inhibitor of the secretory pathway was shown to inhibit peptide transfer to target cells.

Accordingly as hereinbefore mentioned, the present invention provides a method of identifying antigens which are capable of being endogenously processed by a novel cellular secretory pathway and transferred to bystander target cells for presentation to cytotoxic T lymphocytes, comprising: introducing an antigen into a donor cell lacking in MHC class I molecules; incubating in an *in vitro* medium the donor cells, primed cytotoxic T lymphocytes having specificity for the antigen, and target cells which express MHC class I molecules and are labelled with a detectable intracellular marker, under suitable conditions such that the donor cells remain intact, and; measuring the amount of detectable marker released into the incubation medium.

Antigens which require endogenous processing for presentation to CTL are well known. The majority of cytotoxic T lymphocytes recognize epitopes of MHC class I restricted antigens in association with class I major histocompatibility molecules. Such antigens are usually referred to as MHC class I restricted antigens. MHC class I restricted antigens are those antigens which require endogenous cell processing and whose epitopes are presented on the cell surface by MHC class I molecules for recognition by CTL.

Antigens suitable for endogenous processing may be derived from any protein endogenous to a cell, for example from normal, viral, bacterial, defective or tumor proteins. The peptides resulting from endogenous processing of the antigen have the appropriate amino acid sequence for interaction with, and presentation by, MHC class I molecules. The resulting peptides expressed at the cell surface may be detected by CTL and can be extracted from cells, for example by treating whole cells with trifluoroacetic acid. Examples of MHC class I antigens include viral antigens, such as influenza nucleoprotein, vesicular stomatitis virus N protein (VSV-N), tumor antigens, HSV, protozoal, bacterial or parasitic antigens, allopeptides, self peptides, HIV peptides and endogenous peptides and other viral peptides. Examples of MHC class I antigens are provided in Rammensee, et al., 1995 (*Immunogenetics*, 41:178-228).

A cellular secretory pathway for endogenously processing antigens has not heretofore been described. By cellular secretory pathway is meant a route of intracellular antigen processing, whereby endogenous antigen is degraded in the cell and processed into a peptide which is secreted from the cell in a form suitable for interaction with cell surface MHC class I molecules of a bystander target cell and the translocation system. The cellular secretory pathway may be distinguished from the known routes of endogenous antigen processing, which are dependent on the presence of MHC class I molecules in the cell, as the cellular secretory pathway is not dependent on the presence of MHC class I molecules in the antigen processing cell.

- 8 -

The cellular secretory pathway may be further characterized as being dependent on the expression of functional TAP transporters in the cell and it is specifically inhibited by inhibitors of protein secretion, such as Brefeldin A. It will be appreciated that compounds which disrupt the Golgi or interfere with exocytosis of secretory proteins will inhibit protein secretion from the cell. In particular, the cellular secretory pathway is inhibited by Brefeldin A, preferably at concentrations which inhibit protein secretion but do not harm cell viability, for example from 1 to 10 μg/ml, more preferably from 3 to 6 μg/ml, most preferably about 4 to 5 μg/ml.

It should be noted that the sensitivity of the cellular secretory pathway to specific inhibitors of protein secretion such as Brefeldin A, clearly distinguishes the cellular secretory pathway from the pathway for processing exogenous antigens, whereby exogenous antigens may become associated with MHC molecules at the cell surface. The processing of exogenous antigens is not affected by Brefeldin A.

Suitable donor cells for use in the method of the invention, for identifying antigens which are capable of being endogenously processed by a cellular secretory pathway and transferred to bystander target cells for presentation to CTL, are any animal cells, preferably mammalian cells which are lacking in cell surface expression of MHC class I molecules. As noted above, the donor cells lacking in cell surface expression of MHC class I molecules are used in the method of the invention in order to distinguish the novel cellular secretory pathway from the known pathway of endogenous antigen processing, which depends on the presence of MHC class I molecules, whereby peptide processed from the endogenous antigen is transported to the cell surface only in association with MHC class I molecules. However, with suitable controls, it is also anticipated that cells having MHC Class I molecules could be used as donor cells.

Mammalian cells lacking in cell surface expression of MHC class I molecules may be selected by methods known in the art. Suitable cells may lack or have a non-functional defect in the appropriate genes encoding MHC class I molecules, have a mutation in the MHC locus, be unable to transport MHC class I molecules to the cell surface or, be deficient in the expression of class I molecules. It is also anticipated that non-mammalian cells may be used as donor cells, such as avian, amphibian, reptile, fish and insect cells.

Donor cells lacking in cell surface expression of MHC class I molecules may be selected for example by infecting a cell with a recombinant viral vector such as VSV, and testing for lysis by VSV specific CTL. FACS analysis may also be used to detect MHC class I molecules on the surfaces of a putative donor cell. The biosynthesis and intracellular transport of MHC class I molecules may also be biochemically characterized. For example, endo H which cleaves N-linked oligosaccharides only when they are in the high mannose form characteristic of proteins present in the ER and cis-Golgi complex may be used to measure intracellular transport. Pulse-chase methodology may also be utilized to confirm

donor cells lacking in cell surface expression of MHC class I molecules. The above methods are illustrated in part in Restifo, N.P. et al, (1993, *J. Exp. Med.* 177:265).

Examples of cells which express low levels of MHC class I molecules are tumor cells derived from colon, breast, lung mesothelioma and lung cancers of the small cell 5 histology (See Restifo, N.P., supra 1993).

Examples of suitable donor cells include cells lacking in β2-microglobulin, which is required for the intracellular transport of MHC class I molecules to the cell surface. Examples of cells deficient in β2 microglobulin include murine R1E cells and the human Daudi cell line. Tumor cells deficient in cell surface MHC class I molecules may also be used. 10 Suitable donor cells include human T1 (Salter, R.O. and P. Cresswell, 1986, *EMBO* J. 5:943), murine Ltk-(H-2^K), NIH 3T3 (H-2^d), R1.E (Williams et al. 1989, *J. Immunol.* 142:1796). LCL cells with chromosome deletion, T2 cells and mouse cell lines expressing HLA for allotransfer may be used in the methods of the invention.

Preferably, donor cells should be selected which are not defective in other 15 aspects of endogenous antigen processing, for example the proteasome components and the TAP transporters. Cells deficient in certain proteasome or TAP transporter components in addition to MHC class I components may be adapted for use as donor cells by correcting the proteasome or transporter deficiency. For example if the cell is deficient in TAP 1 or TAP 2, such as tumor cells, this defect may be corrected by introducing a nucleotide sequence encoding 20 TAP 1 and/or TAP 2 into the cell.

It is also contemplated that the method of the invention for identifying antigens capable of being processed by a cellular secretory pathway and transferred to bystander target cells may be carried out using donor cells which are not necessarily deficient in surface expression of MHC class I as long as suitable controls are performed. 25 Suitable controls are those which serve to distinguish the novel cellular secretory pathway from the known MHC class I dependent pathway of endogenous antigen processing. Such controls should be performed to rule out the possibility that MHC class I bound antigens at the surface of the donor cell are released into the medium to associate with the target bystander cells. For example, the cells may be pulsed with synthetic peptide and followed 30 for release or antisense nucleotide molecules may be used to inhibit MHC expression. Use of supernatant from VSV infected cells can sensitize cells for VSV specific killing.

The antigen to be identified in the method of the invention may be naturally occurring in the donor cell or may be introduced into the donor cell by methods known in the art. The antigen may be introduced into the cell by microinjection. The antigen may also be 35 introduced into the donor cell by introducing a nucleic acid molecule encoding the antigen into the donor cell under suitable conditions to obtain expression of the antigen in the donor cell.

The nucleic acid molecule encoding an antigen may be introduced into the donor cell under the control of a suitable promoter. The nucleic acid encoding the antigen

may be readily synthesized using techniques known in the art. A nucleic acid molecule encoding an antigen may be isolated and sequenced, for example, by synthesizing cDNAs from RNA and using rapid amplification of cDNA ends (RACE, Frohman, et al., 1988) using oligonucleotides specific for the antigen, and analyzing the sequences of the clones obtained following amplification. Oligonucleotides specific for the antigen may be identified by comparing the nucleic acid sequence to known sequences of the antigen. Nucleic acid molecules encoding the antigen may also be constructed by chemical synthesis and enzymatic ligation reactions using procedures known in the art. The sequence encoding the antigen may also be prepared using recombinant DNA methods.

Nucleic acid molecules having a sequence which codes for the antigen may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the antigen or part thereof. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses, so long as the vector is compatible with the donor cell used.

It is contemplated that the nucleic acid molecules described herein contain the necessary elements for the transcription and translation of the inserted protein antigen-sequence. Suitable transcription and translation elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate transcription and translation elements is dependent on the donor cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the donor cell chosen and the vector employed, other genetic elements, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary transcriptional and translation elements may be supplied by the nucleic acid molecule encoding native antigen.

The nucleic acid molecules may also contain a reporter gene which facilitates the selection of transformed or transfected donor cells. Examples of reporter genes are genes encoding a protein such as β-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. In a preferred embodiment, the reporter gene is *lac* Z. Transcription of the reporter gene is monitored by changes in the concentration of the reporter protein such as β-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. This makes it possible to visualize and assay for expression of the antigen in the donor cell.

Nucleic acid molecules comprising a sequence encoding an antigen can be introduced into donor cells via transformation, transfection, infection, electroporation etc.

Methods for transforming transfecting, etc. host cells to express foreign DNA are well known in the art (see, e.g., Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., *PNAS USA* 75:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216;

5   Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. Molecular Cloning A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, 1989, all of which are incorporated herein by reference).

Suitable expression vectors for directing expression in mammalian donor cells generally include a promoter, as well as other transcriptional and translational control

10   sequences. Common promoters include SV40, MMTV, metallothionein-1, adenovirus Ela, CMV, immediate early, immunoglobulin heavy chain promoter and enhancer, and RSV-LTR. Protocols for the transfection of mammalian cells are well known to those of ordinary skill in the art. Representative methods include calcium phosphate mediated electroporation, retroviral, and protoplast fusion-mediated transfection (see Sambrook et

15   al., supra).

In an embodiment, the nucleic acid molecule may be introduced into the donor cell in a viral vector, preferably a vaccinia viral vector,. most preferably attenuated. Suitable promoters for use with vaccinia viruses include P7.5 (Cochran, M.A. et al, 1985, *J. Virol.* 54:30), P11 (Bertholet, C. et al, 1985, *Proc. Natl. Acad. Sci. USA*, 82:2096), CAE-1

20   (Patel, D.D. et al, 1988, *Proc. Natl. Acad. Sci. USA* 85:9431).

A vaccinia virus containing the nucleic acid molecule may be prepared by homologous recombination. In an embodiment, a nucleic acid molecule encoding a VSV peptide may be ligated into a vaccinia virus shuttle vector such as pJS5. The vector may be transfected into cells that have been infected with vaccinia virus so that the nucleic acid

25   encoding the VSV peptide is inserted into the vaccinia virus by homologous recombination.

The nucleic acid molecule may be inserted into a non-essential site of a vaccinia viral vector. Such non-essential sites are well known and are described, for example, in Perkus et al, 1986, *Virology* 152:285; Hruby et al, 1983, *Proc. Natl. Acad. Sci. USA* 80: 3411 and; Weir and Moss, 1983, *J. Virol.* 46:530. Recombinant viruses expressing the

30   antigen may be readily identified using techniques known in the art and discussed, for example, in Moss, B, 1992, (*Curr. Topics Microbiol. Immunol.* 158:25).

It will be appreciated that the antigen may also be an endogenous antigen which naturally occurs in the donor cell, for example an endogenous tumor antigen. Antigens of intracellular pathogenic agents may be introduced into the donor cell by infecting the

35   donor cell with the pathogenic agent. Examples of intracellular pathogenic agents include viruses, such as HIV, influenza virus, herpes virus, papilloma virus, retroviruses, and rickettsia, and intracellular bacterial pathogens, and protozoan parasites, such as malaria, trypanosomes and *Leishmania*.

Primed CTL having specificity for the antigen may be obtained by known methods. Specific primed cytotoxic T lymphocytes may be obtained from the spleen, blood or lymph of a mammal infected with an intracellular pathogen or having a tumor expressing the antigen of interest.

Primed CTL having specificity for the antigen may also be obtained by immunizing a mammal with the antigen. Methods for immunization of mammals to prime cytotoxic immunity are described for example in Rock, K.L. et al. (1993, *J. Immunol.* 150:1244) and Moss, B. (1990, *Seminars in Immunol.* 2:317). Suitable immunization protocols include administering the antigen or peptide fractions thereof together with β2-microglobulin, or administering a recombinant viral vector encoding the antigen or peptide fractions of the antigen.

Suitable target cells for use in the method of the invention include mammalian cells which express MHC class I molecules on their surface. The target cells and primed CTL may be selected from the same mammal. It will be appreciated that target cells should be selected which do not themselves express the antigen of interest. For example where the antigen of interest is a tumor antigen or an intracellular pathogenic agent of a mammal, a suitable target cell may be selected from non-tumor cells or uninfected cells of the mammal. It will be appreciated that cells, other than those from the mammal may also be used, for example, cells from another animal may be transfected with the appropriate MHC.

Suitable incubation media and conditions for maintaining mammalian cells intact during *in vitro* culture are well known. The target cells are labelled with a detectable intracellular marker. The intracellular marker should be one which is not readily released from the target cell in the absence of cell damage and lysis of the target cell. Suitable intracellular markers include $^{[51]}$Cr, fluorescent markers or Beckman reagent.

An antigen which is endogenously processed by a cellular secretory pathway and transferred to bystander target cells for presentation to cytotoxic T lymphocytes may be identified based on the release of detectable intracellular label into the medium, or based on some other measure of the death of target cells, for example based on the detection of thymidase or DNA fragments.

As hereinbefore noted, the present invention also provides a method of assaying a medium for the presence of a substance that affects processing of an endogenously processed antigen in a cellular secretory pathway, comprising: incubating in an *in vitro* medium a donor mammalian cell having an antigen capable of being endogenously processed by a secretory pathway of the donor cell, a target cell expressing MHC class I molecules and labelled with a detectable intracellular marker, and primed cytotoxic lymphocytes having specificity for the antigen, in the presence or absence of a substance that is suspected of affecting processing of endogenously processed antigen in the cellular secretory pathway,

under suitable conditions such that the donor cells remain intact, and; measuring the amount of detectable marker released into the medium in the presence or absence of the substance.

It will be appreciated that substances which stimulate or inhibit the processing of an endogenously processed antigen in a cellular secretory pathway will, in the method of the invention, result in increased or decreased amounts respectively of detectable marker being released into the medium over a certain time period compared to control incubations carried out in the absence of the substance.

The method may be used to specifically assay for substances that affect processing, rather than secretion, of the endogenously processed antigen in the cellular secretory pathway by simultaneously determining the effect of the substance on secretion of a protein that is known to be secreted from the donor cell, such as growth hormone for example.

The method of the invention may therefore be used to identify substances which inhibit or stimulate processing of antigen from a cell by the cellular secretory pathway. It is anticipated that substances which inhibit or stimulate antigen secretion by the cellular secretory pathway will have an immunomodulating effect when administered to a mammal in vivo and be useful as pharmaceuticals for treating tumors and infection with intracellular pathogens such as viruses.

Substances which inhibit antigen processing may also be useful for the treatment of autoimmune diseases, particularly diseases of MHC class I associated autoimmune disorders such as the HLA-B27 related spondyloarthropathies, including ankylosing spondylitis, Reiter's syndrome and reactive arthropathy, Sjogren's syndrome, rheumatoid arthritis, type I polyendocrine failure, multiple sclerosis, hypothyroidism, Hashimoto's disease, Graves disease and psoriasis vulgaris, and autoimmune diseases resulting from an excessive immune response to foreign antigens of intracellular pathogens. Substances which inhibit antigen secretion may also be useful for the treatment of viral infections where the antigenic load is high, in order to prevent exhaustion and depletion of CTL, leading to the development of immunological tolerance. Substances which inhibit antigen secretion may also be used to treat organs prior to transplant to avoid rejection and to treat mammals with transplanted tissue or organs to decrease the immune rejection response.

Substances which stimulate antigen secretion may be useful for the treatment of tumors and for intracellular pathogens, where the tumor or pathogenic antigens are generally sequestered from the immune system and not appropriately presented on the cell surface by MHC class I molecules.

As hereinbefore mentioned, the invention still further provides a method of characterizing a tumor or viral antigen capable of being endogenously processed by a cellular secretory pathway comprising: obtaining, from a mammal, tumor or virally infected cells and primed cytotoxic T lymphocytes having specificity for the tumor or virally infected

cells; preparing a cDNA library from the tumor or virally infected cells and expressing the cDNA library in transfected host cells; incubating in a medium, transfected host cells, target cells expressing MHC class I molecules and labelled with a detectable intracellular marker and the primed cytotoxic lymphocytes under suitable conditions such that the donor cells

5   remain intact, and; measuring the amount of the detectable marker released into the medium and identifying those transfected cells capable of causing lysis of the target cells and characterizing the transfected DNA of the identified transfected cells.

The mammal from which the tumor cells and primed CTL are obtained may be a mammal having a naturally occurring tumor or a tumor induced by means of a

10   tumorigenic substance, or may be a mammal into which a tumor has been introduced. The mammal may be a human having a tumor and may also be a non-human mammal into which a human tumor or human tumor cell line has been introduced. Human tumor cell lines are well known, see for example Restifo, N. B. et al. (1993) *J. Exp. Med.* 177:265. cDNA expression libraries may be constructed, following known techniques, using mRNA from tumor cells.

15   cDNA clones coding for antigens processed by the cellular secretory pathway may be identified, subcloned and sequenced. The cDNA sequences, or fragments thereof may be used as hybridization probes to isolate genomic DNA encoding for the antigen. The invention therefore provides a method for cloning the gene encoding the tumor antigen and for isolating the antigen. Identification and isolation of the antigen will permit

20   studies of the role of the antigen in the development of the cytotoxic T lymphocyte immune response to the tumor and the development of substances which affect the development of this immunity as stimulators or inhibitors of the immune response. Vaccines may be developed using the genes, for example in retroviral vaccines. The antigens expressed may also be used as the basis for vaccines. It is contemplated that the antigens will bind to the

25   empty MHC 2 molecules which occur on many tumors to aid immune recognition of the tumors.

Nucleic acid molecules encoding tumor antigens may be isolated and sequenced and incorporated into a recombinant molecule which ensures good expression of the antigen or part thereof. In general, a recombinant molecule of the invention contains a nucleic acid molecule, or an oligonucleotide fragment thereof, encoding the antigen and an

30   expression control sequence operatively linked to the nucleic acid molecule or oligonucleotide fragment. A nucleic acid molecule of the invention or an oligonucleotide fragment thereof, may be incorporated into a plasmid vector, for example, pECE. Suitable regulatory elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate regulatory elements is dependent on the host cell

35   chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of regulatory elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other genetic

elements, such as an origin of replication, additional DNA restriction· sites, enhancers, sequences conferring inducibility of transcription, and selectable markers, may be incorporated into the expression vector.

The antigen or parts thereof, may be obtained by expression in a suitable
5   host cell using techniques known in the art. Suitable host cells include prokaryotic or eukaryotic organisms or cell lines, for example, yeast, *E. coli* and mouse NIH 3T3 cells may be used as host cells. The protein or parts thereof may be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution
10   (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

DNA sequences encoding the antigen, or a part thereof, may be expressed by a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, plant, or insect cells. Methods for transforming or transfecting
15   such cells to express foreign DNA are well known in the art (see, e.g., Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., PNAS USA 75:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216; Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. Molecular Cloning A Laboratory Manual, 2nd edition, Cold Spring
20   Harbor Laboratory Press, 1989, all of which are incorporated herein by reference).

Preferably, the tumor antigen is expressed in a mammalian cell. Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) L cells, NIH 3T3 cells and NS-1
25   cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter, as well as other transcriptional and translational control sequences. Common promoters include SV40, MMTV, metallothionein-1, adenovirus Ela, CMV, immediate early, immunoglobulin heavy chain promoter and enhancer, and RSV-LTR. Protocols for the transfection of mammalian cells are well known to those of ordinary skill in
30   the art. Representative methods include calcium phosphate mediated transfection, lipofection-mediated transfection, electroporation, retroviral, and protoplast fusion-mediated transfection (see Sambrook et al., supra).

The tumor antigen may be prepared by culturing the host/vector systems described above, in order to express the recombinant antigen.
35   It will be appreciated that the method of the invention may also be used to characterize antigens, other than tumor antigens, that are capable of being endogenously processed by a cellular secretory pathway. For example, the method will also be useful for characterizing antigens of intracellular pathogens, such as viruses, bacteria and protozoa.

- 16 -

The expression product of the transfected DNA of those transfected host cells capable of causing lysis of the target cells may be directly characterized. Lysis results from CTL recognition of an antigenic peptide presented by the MHC class I molecules on the surface of the target cells. Accordingly, the MHC class I molecules with their associated

5   antigenic peptides may be isolated from the incubation medium and the antigenic peptide extracted using known techniques, as described, for example in Van Bleek, G. M. and S. G. Nathenson, 1990, *Nature* 348:213; and Rammensee et al., 1995, *Immunogenetics*, 41:178-228. The antigenic peptides identified may be the dominant T cell epitope of the antigen.

The MHC Class I fractions may be isolated from the incubation medium by

10   immunoprecipitation and a low molecular weight peptide fraction may be extracted by acid denaturation followed by centrifugation. The low molecular weight peptide fraction may be separated by reverse phase HPLC. The prominent peak corresponding to the antigenic peptide recognised by the T cell receptor may be identified by comparison with the profile obtained from control target cells incubated with non-transfected donor cells. The prominent

15   peak corresponding to the antigenic peptide may be further analyzed, for example by microsequencing. Antigenic peptides may also be identified by peptide scan methods or deletion analysis.

The DNA of those transfected cells capable of causing lysis of the target cells may also be characterized. In an embodiment, all of the transfected host cells capable

20   of causing lysis may be pooled, their DNA extracted and retransfected into host cells for one or more further rounds of screening. Ultimately a single transfected DNA sequence, expressing antigen capable of causing lysis, may be identified, sequenced and expressed for further characterization by sequencing.

The method of the invention thus provides a method for identifying the

25   antigenic peptide or epitope from tumor or viral antigens recognised by the T cell receptor.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

30   The following examples are offered by way of illustration only, and not by way of limitation.

EXAMPLES

Example 1
Virally infected RMA cells transfer peptides to recipient chromium labelled uninfected

35   RMA cells.

A system for investigating surrogate antigen processing was developed using the immunodominant epitope of the VSV-N protein (VSV-N 52-59) presented by the K^b molecules of the MHC class I.

CTL effectors specific for VSV were generated by injecting C57BL/6 mice with VSV (5×10^6 TCID$_{50}$ per site) in the ears and/or foot pads. Five days later the draining lymph nodes were removed and lymphocytes cultured at 4×10^6 cells/ml for 3 days in the absence of stimulation.

Cold targets were prepared as follows: RMA cells were treated with VSV (MOIof 2) for 12 to 18 hours, and subsequently washed three times. Hot targets were prepared as follows: RMA cells were treated with [^{51}]Cr for two hours and subsequently washed three times.

Cold targets were diluted two-fold starting with 2×10^5 cells per well. Each well received 1×10^6 effectors and 1×10^4 hot targets. Effectors, cold and hot targets were incubated together in V-bottom 96-well plates for 4 hours, followed by removal of 100µl of supernatant which was then counted for radioactivity released.

Figure 1A shows the concentration of VSV-N peptide which yields a particular percentage of cytotoxicity. Fifty percent of maximum chromium release was yielded at a peptide concentration of 50 pM. This result was highly reproducible.

In order to determine the RMA dose response, the effector cells were tested against hot RMA target cells pulsed with peptide VSV N52-59 at different concentrations for 2 hours in a dose response.

The effector cell population was tested against hot RMA target cells pulsed with peptide VSV N52-59 at different concentrations for 2 hours in a dose response. The ability of cells to process and transfer the generated peptides to uninfected cells was addressed by infecting cells not labelled with chromium and then incubating these cells together with uninfected target cells which were chromium laden (Figure 1B). When wild-type RMA infected cells were used as the peptide donors we found that uninfected RMA cells were killed efficiently; greater than 25% at a cold/hot of 20/1. It is suggested from our standard curve that the chromium labelled target cells had been exposed to a minimum of 10-50 pM of peptides derived from the VSV infected cells.

In figure 1B, a control in which the surrogate cells were pulsed with a high concentration of synthetic peptide was included to examine if the peptide could be exchanged from the plasma membrane to trigger recognition by T cells on bystander targets. Cells pulsed with saturating concentrations of antigenic peptides were found to not be able to efficiently transfer peptides to recipient cells. Although this may happen over time, as suggested by Huang et al. (A. Y. C. Huang, et al. *Science* 264:961, 1994), we found no evidence for this in our assays.

RMA cells were infected for 12 hours (RMA + VSV) prior to the CTL assay, or alternatively, RMA cells were treated with VSV immediately prior to adding the effectors (RMA.4 + VSV). The effector to target ratios ranged from 12.5:1 to 100:1.

The time frame of the assay (4h) did not allow chromium loaded cells to be infected with VSV, process proteins, and subsequently be recognized by specific CTL (Figure 1C). In addition, our results using cold target competition assays indicate that the peptide transferred was the immunodominant VSV-N 52-59 peptide (data not shown). In summary,

5   virally infected RMA cells were shown to be extremely efficient at transferring peptides to recipient chromium labelled uninfected RMA cells.

Example 2

Ability of human T1 (HLA-A,B,C), murine Ltk- (H-2^k) and NIH 3T3

(H-2^d) cells to transfer peptides to recipient RMA cells

10   In order to examine if the underlying mechanism for transfer involves viral killing or CTL liberation of peptides from infected cells, we studied cells which are not recognized by VSV specific K^b restricted CTL but retain their ability to be infected by virus. We examined the ability of human T1 (R. D. Salter and P. Cresswell. *EMBO J.* 5:943, 1986), murine Ltk$^-$ (H-2^k), and NIH 3T3 cells (H-2^d) to transfer peptides to recipient uninfected

15   RMA (H-2^b) cells.

CTL were generated as described in Example 1 herein, T1, Ltk-(L), NIH3T3 (NIH) and CMT.1-4 cells were treated with or without VSV (MOI of 2) for 12 to 18 hours and subsequently washed 3 times. Hot targets were RMA cells treated with ^{51}Cr for 2 hours and subsequently washed 3 times. The results are shown in Figures 2A and 2C.

20   In order to determine the CTL response T1,L, and NIH + VSV, T1, Ltk-(L), and NIH3T3 (NIH) cells were added to effectors after VSV infection and ^{51}Cr labelling to examine whether or not they are recognized as targets for VSV-specific CTL at different effector to target ratios (E:T). Mock infected Ltk- cells (L+--) were used as negative controls.

In figure 2B, we show that T1, NIH 3T3 and L cells are not lysed by VSV

25   specific K^b restricted CTL. However, each of these cell types can transfer peptides to RMA cells (Figure 2A). Accordingly, the wild-type T1 cells, though not recognised by VSV specific CTL are able to transfer a VSV peptide.

Example 3

Role of TAP transporters and MHC class I molecules in peptide transfer

30   In order to establish the significance of individual components of the antigen processing pathway for peptide transfer, we investigated whether cell variants deficient in one or more elements of this pathway are able to transfer peptide. Human T2 (R. D. Salter and P. Cresswell. *EMBO J.* 5:943, 1986) and murine CMT.64 (H-2^b) cells (Jefferies W. A., et al. *J. Immunol.* 151:2974, 1993 and Gabathuler et al., 1994, J. Exp. Med. 180:1415) do not express

35   either TAP-1 or TAP-2.

The effect of TAP on peptide transfer was investigated as follows. CTL were generated as described in Example 1. Hot targets were RMA cells treated with ^{51}Cr for 2

hours and subsequently washed 3 times. Cold targets serving as peptide donors consisted of RMA, CMT.64+/-IFN-γ (CMT, and CMT.IFN respectively), and CMT.64 cells transfected with the rat TAP 1 and TAP 2 genes (CMT1.2). These cells were treated with VSV (MOI of 2) for 12 to 18 hours and subsequently washed 3 times. Effectors, cold and hot targets were
5   then incubated together in V-bottom 96-well plates for 4 hours, followed by removal of 100 µl of supernatant which was then counted for radioactivity released.

Following infection with VSV, we found that neither the T2 or CMT.64 cell lines were recognized by VSV specific K^b restricted CTL (Jefferies W. A., et al. *J. Immunol.* 151:2974, 1993) (data not shown) and were unable to efficiently transfer peptides to the
10 recipient RMA cells as shown in Figure 3A. As noted above, the wild-type T1 cells, though not recognized by VSV specific CTL (Figure 2B), are able to transfer a VSV peptide (Figure 2A). CMT.64 cells induced with IFN-γ to express TAP-1 and -2 were able to transfer peptide(s) (Figure 3A). Furthermore, CMT.64 transfected with the rat TAP-1 and TAP-2 genes were able to transfer a peptide to RMA cells (Figure 3A). These data demonstrate that
15 infected cells which lack TAP transporters cannot transfer peptide(s). These results reveal a dependency on the expression of the TAP transporters for the exchange of peptide(s) and suggest that the peptides must be translocated into the ER in order to be transferred.

In order to investigate whether interaction with/and transport by MHC class I molecules is a prerequisite for peptide transfer, we examined whether murine R1.E
20 (H-2^k) cells (D. B. Williams, et al. *J. Immunol.* 142:2796, 1989) which lack β-2 microglobulin and therefore do not transport MHC class I heavy chain are able to transfer a VSV peptide.

The effect of heavy and light chains on peptide transfer was investigated as follows. CTL were generated as described in Example 1. Hot targets were RMA cells treated with ^{51}Cr for 2 hours and subsequently washed 3 times. Cold targets consisted of R1E cells
25 and their derivatives R1E transfected with K^b(RIEKb), and R1E transfected with β2 m and K^b(R1EKbb2). Cold targets were treated with or without (RIEKbb2+--) VSV (MOI of 2) for 12 to 18 hours and subsequently washed 3 times. Effectors, cold and hot targets were then incubated together in V-bottom 96-well plates for 4 hours, followed by removal of 100 µl of supernatant which was then counted for radioactivity released. The results are shown in
30 Figure 3B.

R1.E cells were able to efficiently transfer peptides to RMA cells as shown in Figure 3B. The contribution of K^b molecules alone and of K^b and β-2 microglobulin to this peptide transfer was established by examining R1.E cells derivatives transfected with heavy or heavy and light chains. The expression of K^b alone and both K^b and beta-2
35 microglobulin did not affect the efficiency of peptide transfer of transfected R1.E cells (Figure 3B). This indicated that peptide binding and transport of MHC class I molecules was not required for transfer of the peptide.

Example 4

Effect of Brefeldin A on Peptide secretion from infected cells

In order to address whether peptides are secreted from infected cells we used brefeldin A, an inhibitor of protein secretion. Brefeldin A was used at concentrations

5   reported previously (Jefferies W. A., et al. *J. Immunol.* 151:2974, 1993, Nuchtern, J. G., et al. *Nature* 339:223, 1989; Yewdell, J. W. and J. R. Bennink. *Nature* 339:223, 1989) which effectively inhibit protein secretion but does not harm cell viability. Brefeldin A treated RMA cells were infected with VSV and examined for their ability to transfer peptide to recipient cells.

10   The effect of BFA on peptide transfer was investigated as follows. CTL were generated as in Example 1. Hot targets were RMA cells treated with ^{51}Cr for 2 hours and subsequently washed 3 times. Cold RMA cells were treated with RMA.BFA) or without brefeldin A 10μg/ml (BFA) and/or VSV (MOI of 2) for 12 to 18 hours and subsequently washed 3 times. Effectors, cold and hot targets were then incubated together in V-bottom 96

15   well plates for 4 hours, followed by removal of 100 μl of supernatant which was then counted for radioactivity released.

We found that brefeldin A dramatically inhibited peptide transfer as shown in Figure 4, suggesting that the peptide must be actively transported through the secretory pathway for efficient transfer to take place. This was also confirmed by our

20   observation that growth media from Ltk⁻ cells infected with VSV and collected before virally induced lysis could sensitize uninfected RMA cells for CTL recognition.

Example 5

Supernatants from VSV infected Ltk⁻ cells sensitize recipient uninfected RMA cells for VSV specific CTL recognition

25   Cold targets were replaced with supernatant of VSV infected cells in the assay described in Example 1. Supernatant of VSV infected cells was collected 12 hours post infection, before any virally induced lysis, desalted through a G.25 column and lyophilized before reconstitution in 2 ml of medium to achieve a 5 x concentration. The supernatant was added to ^{51}Cr laden RMA cells and the resulting CTL mediated lysis measured. The [51]Cr

30   release was measured by an LKB1282CS Compugamma counter and the specific ^{51}Cr release calculated as (experimental-media control)/(total-media control) x 100%. The spontaneous release never exceeded 20% of the maximum release (treated with 1.5% Triton X-100).

The results are shown in Figure 5, which shows that the supernatant from the VSV infected cells sensitized uninfected RMA cells for CTL recognition. These results

35   indicate that contact between the donor and recipient cells is not necessary for sensitization of target cells for CTL lysis.

Example 6

Ltk cells transfected with VSV-N transfer peptides to recipient non-transfected RMA cells

In order to confirm that the transfer of endogenously processed VSV peptides to recipient target cells for recognition by primed CTL was not an anomaly resulting from the

5   effects of VSV infection of the donor cells, donor cells transfected with VSV-N peptides were prepared. Murine Ltk cells (L cells) were transfected with VSV-N to provide a more controlled and convenient system for the screening of substances that affect the endogenous processing and secretion of antigens.

L cells were transfected as follows. Approximately 1.0μg of DNA encoding

10   VSV-N and the neophosphotransferase gene was transfected into L cells with lipofectin (2 μl). The transfected cells were selected in geneticin (G418) 1mg/ml for 4 days and to half the concentration of G418 thereafter. Isolated clones were selected, grown and frozen. The transfected clones were collected after the cells reached confluency. The supernatants were then checked for their ability to sensitize uninfected target cells for recognition and lysis by

15   VSV specific CTL. The following results, expressed in per cent lysis of RMA target cells, were obtained from 7 different clones: L VSV-N 1 44%, L VSV-N 2 49%, L VSV-N 3 38%, L VSV-N 4 35%, L VSV-N 8 37%, L VSV-N 11 35%, and L VSV-N 13 37%.

The transfection of VSV-N was found to be sufficient to sensitize target cells for CTL killing. Transfected L cells were not recognised directly by the VSV specific cells for

20   CTL killing in the C57Bl/6 mouse (H-2b), but were able to transfer the VSV-N peptide to target cells expressing the K^b molecule. This confirms that endogenous proteins can produce peptides which are secreted and transferred to target cells.

Example 7

Reactivity of CTL after first stimulation

25   The CTL assay described in example 6 was repeated using spleen cells. Spleen cells from mice (C57 Bl/6) infected with VSV were kept in culture for 10 days before restimulation in the presence (spl+) or absence of peptides (spl-). The peptide was VSV-N 52-59 at 1 μM. Restimulation of CTL was done with splenocytes from uninfected C57- Bl/6 mice pulsed with 1 μM of the same peptide (spl-+, spl ++) or without peptide (spl--, spl +-)

30   and irradiated for 30 min. prior to addition for restimulation. Targets for the CTL assay are RMA cells pulsed with 1 μM of VSV-N 52-59 peptides and loaded with [51]Cr. Figure 7A, shows that splenocytes must be primed with the peptide and restimulated with peptide pulsed cells in order to recognize efficiently VSV-N 52-59 specific targets. Figures 7B, 7C and 7D show that peptides are necessary and must be present for restimulation and in the

35   previous incubation for the generation of specific CTL.

Example 8

Peptide transer is not due to VSV replication pathway

In order to demonstrate that the life cycle of VSV was not specifically leading to the release of the VSV-N 52-59 peptide, a recombinant vaccinia virus encoding
5   the VSV-N-52-59 peptide was prepared.

Vaccinia Virus Constructs

The recombinant Vaccinia Virus containing the VSV-N 52-59 peptide gene (VV-N) was constructed by first ligating a double stranded synthetic oligonucleotide encoding the VSV-N 52-59 peptide into a Vaccinia Virus shuttle vector pJS5 (provided by
10   Dr. B. Moss, NIH, Bethesda, MD). The oligonucleotide, which contained SacI and NheI overhangs for ligation into pJS5, is as follows: 3′-tcgag-tac-tct-cct-ata-cag-atg-gtt-ccg-gag-act-cgatc-5′ with the complementary strand as 5′-c-atg-aga-gga-tat-gtc-tac-caa-ggc-ctc-tga-g-3′. The VV-N peptide was then made by homologous recombination when the pJS5-VSVN52-59 construct was transfected into CV-1 cells infected with Vaccinia Virus (WR
15   strain provided by Dr. S. Gillam, Vancouver,B.C.). VV-N peptide was plaque purified three times in BS-C-1 cells, then amplified in Hela S3 cells before virus purification on a sucrose gradient (Earl and Moss, 1992). The shuttle vector pJS5 without an insert was also cloned into Vaccinia Virus (VV-pJS5) to provide a vector only control.

Vaccinia Virus Infection

20   Vaccinia virus infection of cold targets was done as follows. VV-N peptide and VV-pJS5 were trypsinized in 0.1 volume 2.5 mg/ml trypsin (Difco 1:250) for 30 minutes at 37°C, vortexing every 10 minutes. After washing CMT.64, CMT.12-12, and L cells with phosphate buffered saline (PBS) the virus was added to the cells (MOI of 10) along with 0.5 ml RPMI supplemented with 2% heat inactivated FBS, glutamine, penicillin, streptomycin
25   and Hepes. They were incubated 1.5 hours at 37°C before adding 3 ml RPMI supplemented with 10% heat inactivated FBS, glutamine, penicillin, streptomycin and Hepes. The cells were removed with versene and 0.5% trypsin, washed 2 times with media before addition to the CTL assay. The total infection time was 6 hours until the start of the assay or longer as indicated. Hot targets, which were RMA cells or as indicated, were treated with ^{51}Cr for 1
30   hour at 37°C and subsequently washed. [51]Cr was used at 100mCi per 1x106 cells. Cold targets were diluted two-fold or three-fold when indicated starting with 2×10^5 cells per well at 20:1 ratio cold versus hot targets. Each well received 1×10^6 effector CTL and 1×10^4 hot targets (100:1 ratio effector versus : hot targets). Effectors plus cold and hot targets were incubated together in V-bottom 96-well plates for 4 hours for radioactivity release. In some
35   assays, where indicated, cold targets are replaced by medium containing peptides or viruses. The supernatant of VSV infected cells was previously desalted through a G.25 column and lyophilized before reconstitution in 2 ml of medium to achieve a 5 x concentration. The ^{51}Cr release was measured by an LKB1282CS Compugamma counter and the specific ^{51}Cr release

calculated as (experimental-media control)/(total-media control) x 100%. The spontaneous release never exceeded 20% of the maximum release (treated with 1.5% Triton X-100).

In the initial experiment, Ltk⁻ (H-2^k) were infected and examined for the ability of VSV, vaccinia virus (VV) and vaccinia virus encoding VSV-NP 52-59 to transfer
5   the VSV-N peptide to recipient cells. The results, shown in Figure 8A, demonstrate that only VSV itself and the VV-N peptide construct are able to transfer the peptide to RMA cells. The activities of both viruses are titratable and the VSV appears to be more efficient than the recombinant VV at transferring the peptide. These results demonstrate that in a heterologous system, VSV-N peptide expression results in peptide transfer. These results
10   also demonstrates that VSV-N peptide expression is not dependent on the expression of the H-2b haplotype. Furthermore, this data also demonstrates that an 8 amino acid peptide can transfer the activity. Thus it is not dependent on extracellular cleavage events.

In order to investigate the TAP dependency of peptide transfer in the heterologous expression system, CMT.64 or CMT.64 cells expressing TAP-1/2 (CMT.12-12)
15   were infected with the VV-N peptide or with the wild-type VV. The results, shown in Figure 8B, demonstrate the TAP dependency of the transfer of VSV-N peptide expressed in a heterologous system. These data confirm that the peculiarities of the VSV life cycle do not contribute to the peptide transfer mechanism.

The results using a vaccinia virus construct expressing the VSV-N peptide
20   epitope demonstrate clearly that the peptide transfer is not the result of the VSV replication pathway. The vaccinia virus encoded peptide (VSV-N 52-59) also requires a functional TAP transporter, strongly supporting the view that the normal antigen peptide processing machinery is required. In addition, these data demonstrate that the 8 aa peptide itself can be transferred and transfer is thus not dependent on the proteolytic cleavage of a
25   larger fragment extra or intracellularly. Overall, peptide transfer is not dependent on VSV infection, or the viral lytic cycle.

WE CLAIM:

1.   A method of identifying antigens which are capable of being endogenously processed by a cellular secretory pathway and transferred to bystander target cells for presentation to cytotoxic T lymphocytes, comprising: introducing an antigen into a donor cell lacking in MHC class I molecules; incubating in an *in vitro* medium the donor cells, primed cytotoxic T lymphocytes having specificity for the antigen, and target cells which express MHC class I molecules and are labelled with a detectable intracellular marker, under suitable conditions such that the donor cells remain intact, and; measuring the amount of detectable marker released into the incubation medium.

2.   A method as claimed in claim 1 wherein the antigen is a viral, bacterial, protozoan, or tumor antigen.

3.   A method as claimed in claim 1 wherein the antigen is a self antigen or an alloantigen.

4.   A method as claimed in claim 2 wherein the antigen is VSV.

5.   A method as claimed in claim 1 wherein the donor cell is transfected with a nucleic acid molecule encoding the antigen.

6.   A method as claimed in claim 1 wherein the donor cell is R1.E(H-2^k) or a Daudi cell.

7.   A method as claimed in claim 1 wherein the detectable marker is [51]chromium.

8.   A method of assaying a medium for the presence of a substance that affects processing of an endogenously processed antigen in a cellular secretory pathway, comprising: incubating in an *in vitro* medium a donor mammalian cell having an antigen capable of being endogenously processed by a secretory pathway of the donor cell, a target cell expressing MHC class I molecules and labelled with a detectable intracellular marker, and primed cytotoxic lymphocytes having specificity for the antigen, in the presence or absence of a substance that is suspected of affecting processing of endogenously processed antigen in the cellular secretory pathway, under suitable conditions such that the donor cells remain intact, and; measuring the amount of detectable marker released into the medium in the presence or absence of the substance.

9.   A method as claimed in claim 8 wherein the donor cell lacks MHC class I molecules.

10.   A method as claimed in claim 9 wherein the antigen is VSV-N protein and the donor cell is RMA.

5   11.   A method of characterizing a tumor or viral antigen capable of being endogenously processed by a cellular secretory pathway comprising: obtaining, from a mammal, tumor cells or virally infected cells and primed cytotoxic T lymphocytes having specificity for the tumor or virally infected cells; preparing a cDNA library from the tumor or virally infected cells and expressing the cDNA library in transfected host cells;

10   incubating in a medium, transfected host cells, target cells expressing MHC class I molecules and labelled with a detectable intracellular marker and the primed cytotoxic lymphocytes under suitable conditions such that the donor cells remain intact, and; measuring the amount of the detectable marker released into the medium and identifying those transfected cells capable of causing lysis of the target cells and characterizing the transfected DNA or

15   expression product of the transfected DNA of the identified transfected cells.

FIGURE 1A

FIGURE 1B

FIGURE 1C

4/15
FIGURE 2A

5/15
FIGURE 2B

FIGURE 2C

Peptide exchange (RMA as Hot Targets)

Legend:
- ■ R VSV
- ▨ RS VSV
- ▦ R VSV BFA
- ▨ C1.4 VSV
- □ C VSV
- ■ R p

y-axis: % sp. rel. (0 to 40)

x-axis: C:H (20, 10, 5, 2.5)

FIGURE 3A

FIGURE 3B

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7A

(1) CTL assay with spleen cells - MC I --
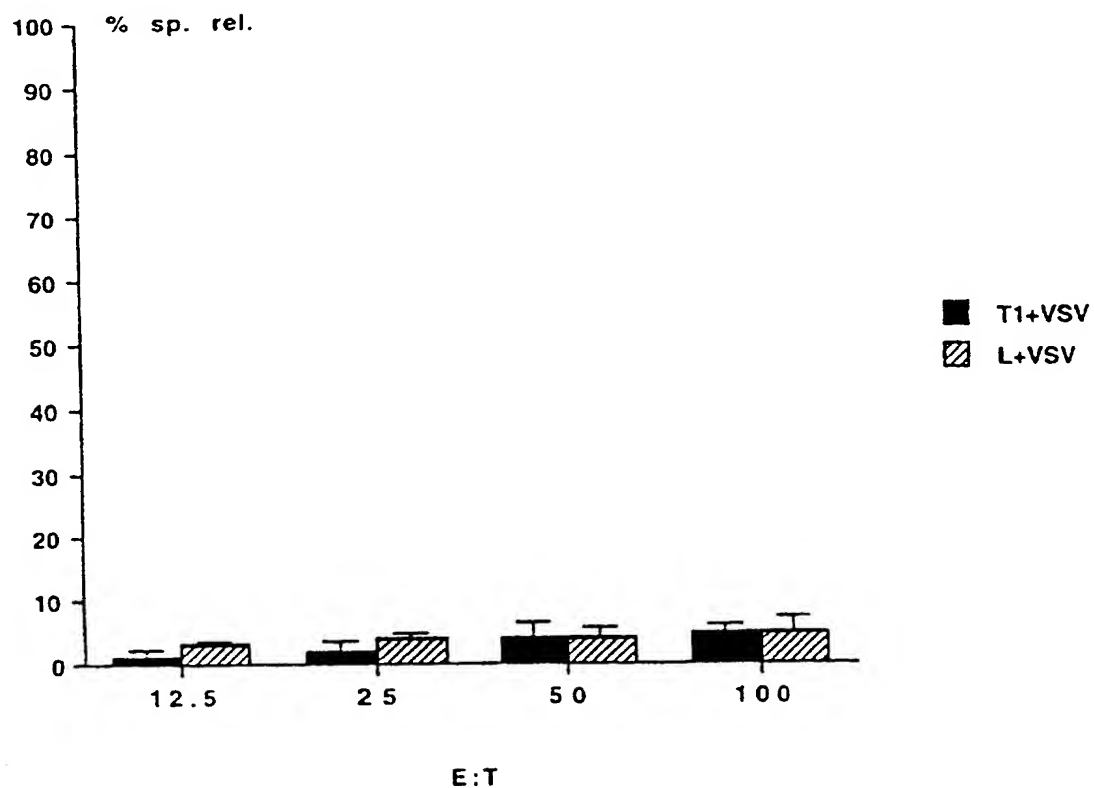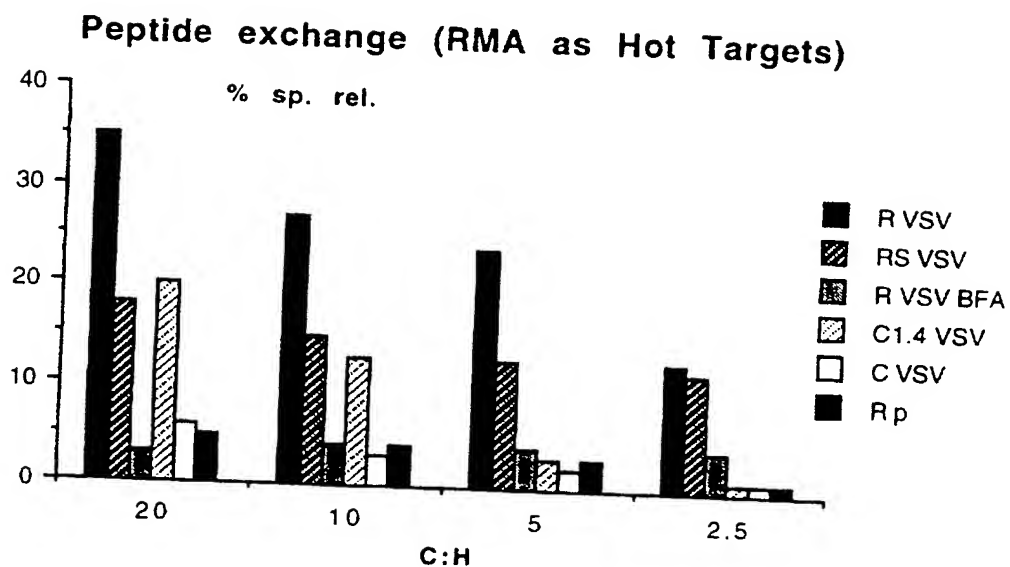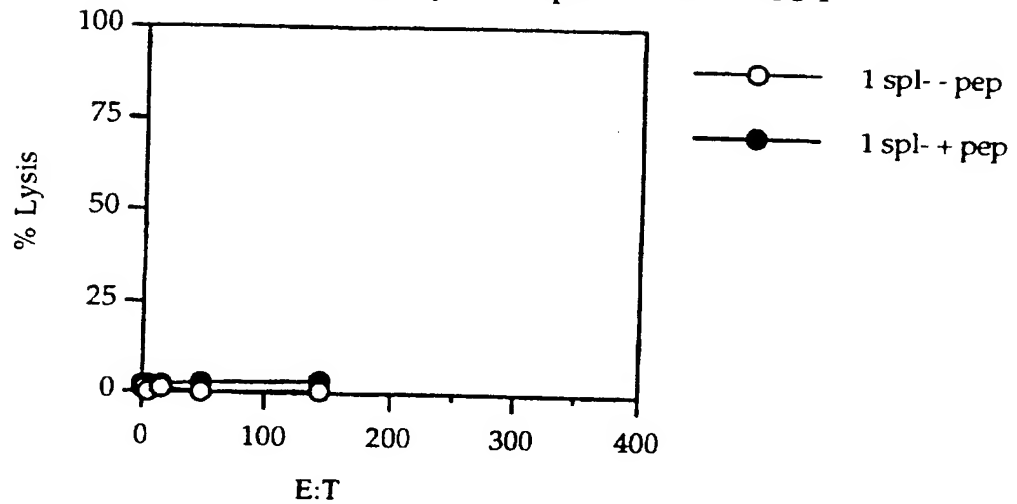
FIGURE 7B

(2) CTL assay with spleen cells - MCI ++

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13/15

FIGURE 7C
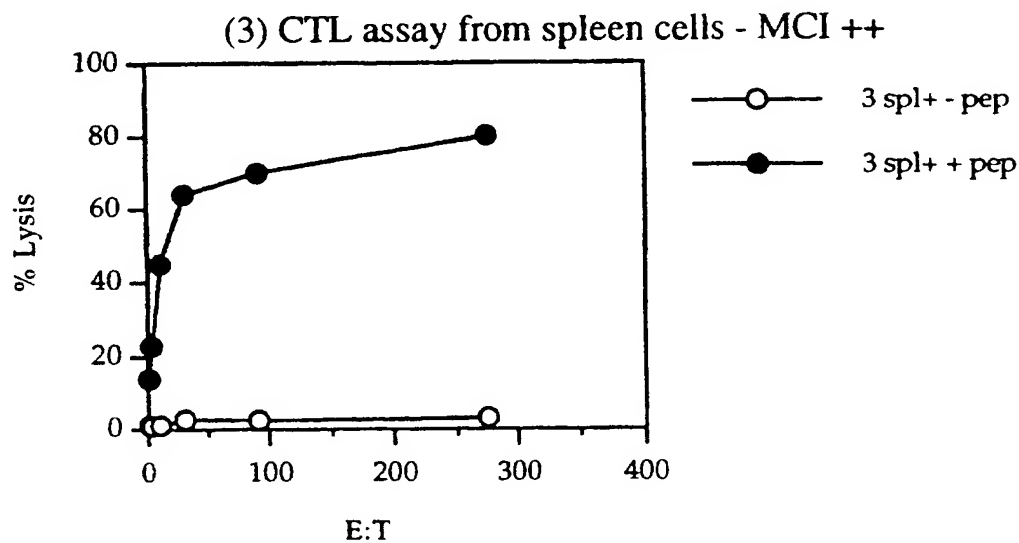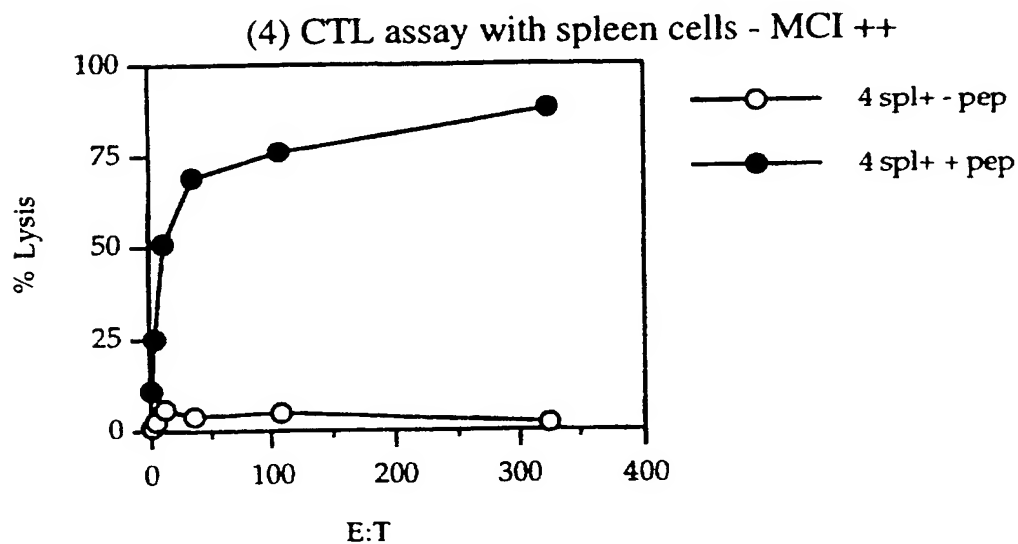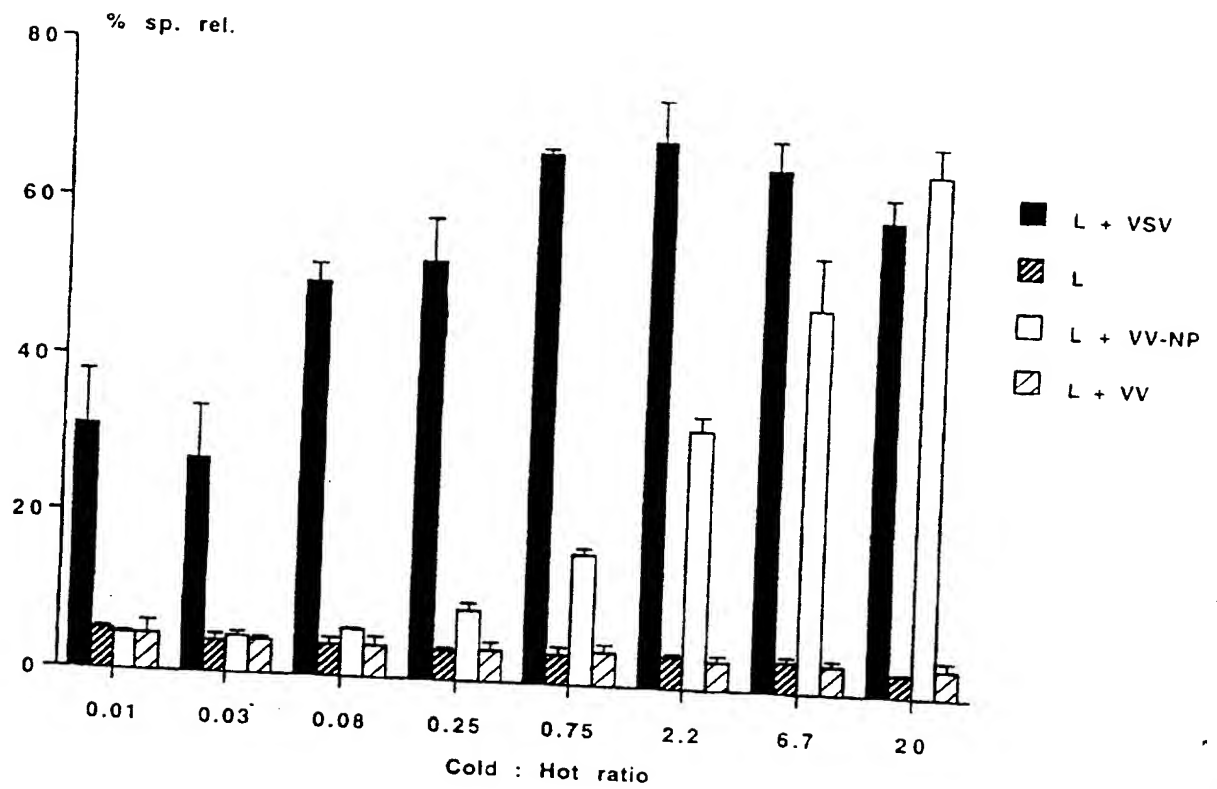
(3) CTL assay from spleen cells - MCI ++

○ 3 spl+ - pep
● 3 spl+ + pep

% Lysis

E:T

FIGURE 7D

(4) CTL assay with spleen cells - MCI ++

○ 4 spl+ - pep
● 4 spl+ + pep

% Lysis

E:T

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14/15
FIGURE 8A

FIGURE 8B
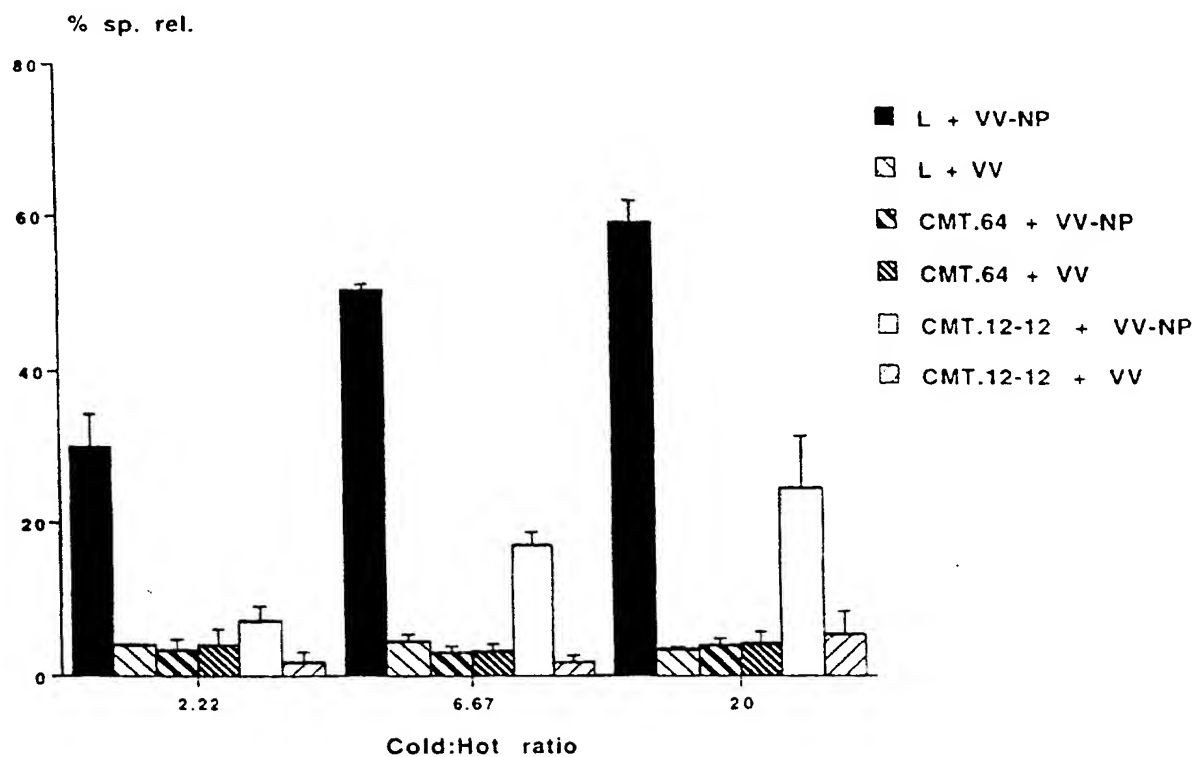
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/50   G01N33/569   G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 175, no. 3, 1 March 1992, pages 719-729, XP002035165 N. A. HOSKEN ET AL.: "An endogenous antigenic peptide bypasses the class I antigen presentation defect in RMA-S" see abstract see page 720	1-11
X	EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 23, 1993, pages 1796-1801, XP002035166 X. ZHOU ET AL.: "TAP2-defective RMA-S cells present Sendai virus antigen to cytotoxic T lymphocytes." see the whole document	1-11

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 1997	2 1 -07- 1997

Name and mailing address of the ISA	Authorized officer
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page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category *	Citation of document, with indication, where appropriate, of the relevant passages	
A	JOURNAL OF CELLULAR BIOCHEMISTRY, vol. suppl 16D, 5 - 27 March 1992, page 17 XP002035167 F. LÉVY ET AL.: "Transport of peptides across the Er membrane and assembly of MHC class I. " ---	
P,A	WO 96 09380 A (THE UNIVERSITY OF BRITISH COLUMBIA) 28 March 1996 -----	

1

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9609380 A	28-03-96	NONE	

1/15
FIGURE 1A

FIGURE 1B

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FIGURE 1C

FIGURE 2A

FIGURE 2B

FIGURE 2C

Peptide exchange (RMA as Hot Targets)

FIGURE 3A

FIGURE 3B

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7A

(1) CTL assay with spleen cells - MC I --

FIGURE 7B

(2) CTL assay with spleen cells - MCI ++

FIGURE 7C

(3) CTL assay from spleen cells - MCI ++

Legend:
- ○ 3 spl+ - pep
- ● 3 spl+ + pep

FIGURE 7D

(4) CTL assay with spleen cells - MCI ++

Legend:
- ○ 4 spl+ - pep
- ● 4 spl+ + pep

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14/15
FIGURE 8A

FIGURE 8B